

Burkholderia cepacia Complex Phage-Antibiotic Synergy (PAS): Antibiotics Stimulate Lytic Phage Activity

Fatima Kamal, Jonathan J. Dennis

Centennial Centre for Interdisciplinary Science, Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada

The *Burkholderia cepacia* complex (Bcc) is a group of at least 18 species of Gram-negative opportunistic pathogens that can cause chronic lung infection in cystic fibrosis (CF) patients. Bcc organisms possess high levels of innate antimicrobial resistance, and alternative therapeutic strategies are urgently needed. One proposed alternative treatment is phage therapy, the therapeutic application of bacterial viruses (or bacteriophages). Recently, some phages have been observed to form larger plaques in the presence of sublethal concentrations of certain antibiotics; this effect has been termed phage-antibiotic synergy (PAS). Those reports suggest that some antibiotics stimulate increased production of phages under certain conditions. The aim of this study is to examine PAS in phages that infect *Burkholderia cenocepacia* strains C6433 and K56-2. Bcc phages KS12 and KS14 were tested for PAS, using 6 antibiotics representing 4 different drug classes. Of the antibiotics tested, the most pronounced effects were observed for meropenem, ciprofloxacin, and tetracycline. When grown with subinhibitory concentrations of these three antibiotics, cells developed a chain-like arrangement, an elongated morphology, and a clustered arrangement, respectively. When treated with progressively higher antibiotic concentrations, both the sizes of plaques and phage titers increased, up to a maximum. *B. cenocepacia* K56-2-infected *Galleria mellonella* larvae treated with phage KS12 and low-dose meropenem demonstrated increased survival over controls treated with KS12 or antibiotic alone. These results suggest that antibiotics can be combined with phages to stimulate increased phage production and/or activity and thus improve the efficacy of bacterial killing.

The *Burkholderia cepacia* complex (Bcc) is a group of Gram-negative bacterial opportunistic pathogens that can cause chronic lung infection in cystic fibrosis (CF) and immunocompromised patients (1–3). Currently, there are 18 species that have been identified as members of the Bcc (4–6). Among them, *Burkholderia cenocepacia* and *Burkholderia multivorans* are most associated with CF patients, accounting for ~34% and 30% of all Bcc infections, respectively (7, 8). In up to 20% of patients, Bcc colonization may be associated with rapid pulmonary deterioration, leading to death by an invasive infection termed “cepacia syndrome” (2, 3, 9). It is difficult to eliminate infections caused by Bcc bacteria because of their high levels of innate resistance to both antibiotics (10) and biocides (11) and their ability to form biofilms (12). Furthermore, Bcc bacteria can spread between people (13–15) and can survive in respiratory droplets on surfaces (16). Clinical treatment of Bcc-infected patients typically involves combination therapy with three or more antibiotics (17–19), even though synergistic activity is rarely observed (19). Only 23 to 38% of clinical Bcc isolates are significantly inhibited by ceftazidime, meropenem, and minocycline, antibiotics commonly used to treat Bcc infections (19).

Because Bcc bacterial infections in CF patients are highly resistant to and cannot be cleared by antibiotics, all antibiotic therapy is rendered subinhibitory. One proposed alternative treatment strategy is that of phage therapy (20). Most phages are extremely specific and target only a subset of bacterial cells without affecting a patient’s normal microflora. Phages also replicate exponentially in bacterial hosts, which enhances their therapeutic potential for treating infections. Phage therapy was commercially developed in the 1930s but was abandoned in Western countries following the discovery and widespread introduction of broad-spectrum chemical antibiotics (20, 21). With the recent emergence of multidrug-resistant (MDR) and extremely drug-resistant (XDR) bacteria, including those of the Bcc, there has been renewed interest in phage

therapy. In a type of coevolutionary arms race, phages can rapidly adapt to counter improvements in bacterial resistance, which is in direct contrast to the long development time required for new static chemical antibiotics (21). Recent studies exploring the treatment of Bcc infections via phage therapy have demonstrated promise (22–26), including our recent study showing Bcc phage efficacy in a mouse infection model (26).

How phages behave in the presence of chemical antibiotics has been investigated previously. It has been reported that more phages are produced from bacteria in the presence of penicillin than in its absence (27–30). There have also been reports of the stimulation of phage development in *Escherichia coli* (31) and *Staphylococcus aureus* (32) by β -lactam antibiotics. More recently, some phages have been observed to form larger plaques in the presence of sublethal concentrations of certain antibiotics; this effect has been termed phage-antibiotic synergy (PAS) (33). PAS has shown promise in the killing of *Pseudomonas aeruginosa* (34) and in the eradication of *E. coli* biofilms (35). Those reports suggest that some chemical antibiotics have the ability to stimulate increased production of phages under certain conditions. In this study, we demonstrate that some antibiotics appear to induce increased production of several Bcc phages. We extend these find-

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Address correspondence to Jonathan J. Dennis, jon.dennis@ualberta.ca.

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ings to show PAS against members of the Bcc in an *in vivo* infection model. *Galleria mellonella* larvae have previously been used for studies of pathogenic bacteria such as *P. aeruginosa* (36), *Bacillus cereus* (37), *Francisella tularensis* (38), and members of the Bcc (39) and also to test phage therapy against clinically relevant Bcc strains (22).

MATERIALS AND METHODS

Bacterial strains and phages. The Bcc bacterial strains used in this study were obtained from the *Burkholderia cepacia* complex experimental strain panel (40, 41). Bacterial cells were grown overnight in 10 ml one-half-strength Luria-Bertani (1/2 LB) broth at 30°C and at 220 rpm in a gyratory shaker. The cells were diluted 1:100 in fresh medium and grown an additional 3.5 h to an optical density at 600 nm (OD_{600}) corresponding to exponential phase and a titer of $\sim 2 \times 10^8$ CFU. Optical density values were measured by using a Victor X3 spectrophotometric plate reader (PerkinElmer, Woodbridge, ON, Canada). Phages KS12 and KS14 were previously isolated and characterized by members of the Dennis laboratory, and those results were previously reported (22, 26, 42). Both phages KS12 and KS14 contain double-stranded DNA nucleic acids and are of the phage family *Myoviridae* (A1 morphotype). Phage KS14 (vB_BceM-KS14), isolated from a *Dracaena* sp. (dragon tree) soil extract plated onto *Burkholderia multivorans* C5393, is a subfamily *Peduvirinae*, genus “P2-like” virus (42) with a genome size of 32,317 bp, encoding 44 proteins. KS14 can lysogenize host cells as a plasmid (42). Phage KS12 is a myovirus isolated from soil planted to *Diets grandiflora* (wild iris) on *B. cenocepacia* K56-2, and its genomic content is currently unknown (22). Based upon experimental data (not shown), KS12 has been demonstrated to not lysogenize any of its known Bcc hosts. Phage titers were determined by using the double-layer agar technique (43). Briefly, 100 μ l of phage was added to 100 μ l of exponential-phase bacterial cells and mixed in 3 ml top agar that was poured onto 20-ml agar plates. Plates were incubated overnight at 30°C, and plaques were identified and examined by visualization.

Media and antibiotics. Bacterial strains were grown in 1/2 LB broth. For use with a double-layer agar (DLA) phage plaquing method (43), this same medium was supplemented with Select agar (Invitrogen, Burlington, ON, Canada) at final concentrations of 0.4% and 1.4% in the top and bottom layers, respectively. Ciprofloxacin, tetracycline, minocycline, levofloxacin, and ceftazidime were purchased from Sigma-Aldrich Inc. (St. Louis, MO), and meropenem was purchased from AstraZeneca Canada Inc. (Mississauga, ON, Canada). MICs of antibiotics were determined by using a 96-well microplate dilution protocol (44). Briefly, 5 μ l of exponential-phase cells was added to 100 μ l of antibiotic prepared in Mueller-Hinton broth and grown overnight at 30°C and at 220 rpm in a gyratory shaker. The MIC of an antibiotic for a specific bacterial strain was determined to be the concentration of antibiotic at which the optical density (OD_{600}) was equal to that of a cell-free blank control.

Effects of different concentrations of antibiotics. For measurement of plaque sizes and phage particle number determinations in the presence of antibiotics, different concentrations of antibiotics were added to top agar in a double-layer agar assay. The antibiotic concentrations for phage KS12 ranged from 0.625 μ g/ml to 10 μ g/ml ciprofloxacin, 2.5 μ g/ml to 40 μ g/ml meropenem, and 2.5 μ g/ml to 40 μ g/ml tetracycline. Similarly, the antibiotic concentrations used for phage KS14 were 62.5 μ g/ml to 1 mg/ml ciprofloxacin, 2.5 μ g/ml to 40 μ g/ml meropenem, and 25 μ g/ml to 100 μ g/ml tetracycline. These concentrations correspond to 1/4 \times MIC to 4 \times MIC of antibiotics for the bacterial hosts *B. cenocepacia* K56-2 and C6433. As a control, 100 μ l of sterile water was added in place of the antibiotic suspension to account for agar dilution effects. Phage plaques were backlit and viewed under the magnifying glass of a New Brunswick Scientific colony counter (model C110), and plaque size was measured by using digital calipers manufactured by Tresna (Guilin, China). For phage particle counts, 5 ml of 1/2 LB broth was added to each plate, and the top agar layer was scraped off and added to 15-ml tubes. Phages were released into liquid medium by gentle pipetting, solid debris was removed by low-

speed centrifugation, and the supernatant was filtered by using EMD Millipore (Etobicoke, ON, Canada) 0.22- μ m syringe filters. Filtrates were serially diluted and used in double-layer agar assays. Differences in phage plaque sizes, phage titers, numbers of surviving bacterial cells, and numbers of surviving waxworm larvae were statistically analyzed by using one-way analysis of variance (ANOVA), compared to controls. An ANOVA with Dunnett's posttest was used, with a *P* value of <0.05 being considered statistically significant. The analyses were performed by using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA).

To ensure that bacterial numbers were similar for the different concentrations of antibiotics tested, bacterial cells were initially grown to exponential phase. One milliliter of bacterial cells was added to different subinhibitory concentrations of antibiotics in 15-ml tubes and grown for an additional 6 h. The antibiotic concentrations used were 1.25 μ g/ml ciprofloxacin, 5 μ g/ml meropenem, and 5.5 μ g/ml tetracycline. Cell counts were determined by taking samples at different time points and plating the samples after serial dilution. For analysis of bacterial cells in the presence of different concentrations of antibiotics, 100 μ l of cells grown overnight at 30°C was added to 3 ml of top agar containing subinhibitory concentrations of antibiotics and spread onto 20-ml agar plates. The plates were incubated overnight at 30°C, and cells were scraped and suspended in 4% paraformaldehyde in phosphate-buffered saline (PBS). Cells were stained with 2% phosphotungstic acid, and transmission electron micrographs were taken by using an FEI/Philips (Hillsboro, OR) Morgagni transmission electron microscope with a Gatan digital camera to visualize the effects of different concentrations of antibiotics.

Combination treatment of *B. cenocepacia*-infected *Galleria mellonella* larvae. *G. mellonella* larvae were purchased from Recorp Inc. (Georgetown, ON, Canada). Larvae were stored in wood chips at 4°C. A 250- μ l Hamilton syringe was used to inject 5- μ l aliquots of *B. cenocepacia* K56-2 into *G. mellonella* larvae, as previously described (39). *B. cenocepacia* K56-2 bacteria suspended in 10 mM $MgSO_4$ plus 1.2 mg/ml ampicillin were injected into larvae via the last left proleg, followed by injection of phage at a multiplicity of infection (MOI) of 100, or 0.031 μ g meropenem or 1.5 μ g minocycline resuspended in 5 μ l sterile 10 mM $MgSO_4$ was injected in the next prolegs through separate injections. As *G. mellonella* larvae contain ~ 50 μ l of hemolymph, we estimate that the final concentration in the larvae was 6 μ g/ml meropenem or 30 μ g/ml minocycline alone or in combination with phage KS12. Control larvae were injected with 5 μ l of only 10 mM $MgSO_4$ plus 1.2 mg/ml ampicillin. Larvae were placed in the dark in an incubator at 30°C. Ten larvae were injected for each treatment group, and larvae were scored as dead or alive at 48 and 72 h postinfection (p.i.). Larvae were assessed to be expired if they did not respond to touch with movement.

Antibiotic-resistant mutants. Plasmid pBBR1MCS-3 (45) carrying a tetracycline resistance marker was electroporated into K56-2 cells, and transformed resistant cells were selected on medium containing tetracycline. In addition, wild-type K56-2 cells grown overnight with high concentrations of tetracycline in the medium produced spontaneous mutants that were isolated. These resistant cells were grown overnight at 30°C and at 220 rpm in 1/2 LB broth with tetracycline and tested in PAS experiments. One hundred microliters of phage was added to 100 μ l exponential-phase cells, mixed with 3 ml of top agar containing different concentrations of antibiotics, and plated by using the DLA method. Plaque sizes were measured as described above.

RESULTS

Effects of different concentrations of antibiotics on Bcc PAS.

With the objective of developing phage therapy as an alternative treatment strategy for the Bcc, phages KS12 and KS14 were tested against *Burkholderia cenocepacia* strains K56-2 and C6433, respectively. The phages were initially tested for PAS in the presence of different concentrations of several different classes of antibiotics, including ampicillin, ceftazidime, ciprofloxacin, kanamycin, levofloxacin, meropenem, minocycline, piperacillin, and tetracycline.

TABLE 1 Antibiotic MICs for *B. cenocepacia* strains

<i>B. cenocepacia</i> strain	MIC (μg/ml) ^a					
	CIP	MEM	TET	MIN	CAZ	LEV
C6433	250	10	100	0.125	250	125
K56-2	2.5	10	10	0.25	500	15

^a CIP, ciprofloxacin; MEM, meropenem; TET, tetracycline; MIN, minocycline; CAZ, ceftazidime; LEV, levofloxacin.

A PAS effect with phages KS12 and KS14 was observed for only six of these antibiotics: ciprofloxacin, meropenem, tetracycline, minocycline, levofloxacin, and ceftazidime. Bacterial cells were grown to exponential phase, and the MICs of the antibiotics were determined. As shown in Table 1, strain C6433 MICs ranged from 0.125 μg/ml for minocycline to 250 μg/ml for ciprofloxacin and ceftazidime, and strain K56-2 MICs ranged from 0.25 μg/ml for minocycline to 500 μg/ml for ceftazidime.

Bcc phage KS12 and KS14 plaque sizes and titers were determined on K56-2 and C6433 cells, respectively. The phages showed an increase in plaque size in the presence of different concentrations of antibiotics, as shown in Table 2, with a maximum increase in plaque size observed for meropenem, a β-lactam antibiotic. The increase in plaque size was concentration dependent up to a maximum size. For phage KS12, the plaque diameter size increased from 1.22 mm without antibiotics to 2.37 mm when 4× MIC of meropenem was added to the medium, an increase of 97.1% (equal to a 94.3% increase in area). For phage KS12 plated onto *B. cenocepacia* K56-2 cells in the presence of various concentrations of antibiotics (0.625 μg/ml to 10 μg/ml ciprofloxacin, 2.5 μg/ml to 40 μg/ml meropenem, or 2.5 μg/ml to 40 μg/ml tetracycline), these differences were statistically significant. For KS12 in the presence of 10 μg/ml ciprofloxacin, one-way ANOVA with Dunnett’s posttest was used to calculate the probability of a null hypothesis, that these plaque sizes are the same, as a *P* value of <0.0001. Similarly, for 40 μg/ml meropenem, the *P* value was <0.0001, and for 40 μg/ml tetracycline, the *P* value was <0.0001. Phage KS12 plaque size increases were also observed for 1× MIC of minocycline (1.37 ± 0.13 mm; *P* < 0.01), 1/2× MIC of ceftazidime (1.67 ± 0.12 mm; *P* < 0.0001), and 1/2× MIC of levofloxacin (1.54 ± 0.20 mm; *P* < 0.0001).

Phage KS14 showed a plaque diameter increase from 2.81 mm

in the absence of antibiotics to 4.35 mm in the presence of 4× MIC of meropenem, an increase of 77.4% (equal to a 54.8% increase in area). For phage KS14 plated onto *B. cenocepacia* C6433 cells in the presence of various concentrations of antibiotics (62.5 μg/ml to 1 mg/ml ciprofloxacin, 2.5 μg/ml to 40 μg/ml meropenem, or 25 μg/ml to 100 μg/ml tetracycline), significant differences (one-way ANOVA with Dunnett’s posttest) were observed for 1 mg/ml ciprofloxacin (*P* < 0.0001), 40 μg/ml meropenem (*P* < 0.0001), and 100 μg/ml tetracycline (*P* < 0.0001). In addition, KS14 plaque size increases were also observed for 1× MIC of minocycline (3.64 ± 0.32 mm; *P* < 0.0001), 1/4× MIC of ceftazidime (3.19 ± 0.22 mm; *P* < 0.001), and 1/2× MIC of levofloxacin (3.47 ± 0.17 mm; *P* < 0.0001).

Phage titers also showed an increase with increasing concentrations of antibiotics. As shown in Table 3, phage KS12 titers were increased in the presence of 5 μg/ml ciprofloxacin (2× MIC), 20 μg/ml meropenem (2× MIC), and 20 μg/ml tetracycline (2× MIC), compared to controls not containing antibiotics. However, none of these increases in phage KS12 mean titers were statistically significant. Similarly, KS12 mean titers did not significantly increase in the presence of 1/2× MIC of ceftazidime (6.00 ± 0.26 log CFU/ml) or levofloxacin (6.07 ± 0.22 log CFU/ml) and were significantly reduced in the presence of 1× MIC of minocycline (4.80 ± 0.35 log CFU/ml; *P* < 0.01). In contrast, as also shown in Table 3, phage KS14 titers were significantly increased with 500 μg/ml ciprofloxacin (2× MIC; *P* < 0.01), 20 μg/ml meropenem (2× MIC; *P* < 0.0001), and 100 μg/ml tetracycline (1× MIC; *P* < 0.01). No change in KS14 mean titers was found with 1/4× MIC of ceftazidime (7.22 ± 0.51 log CFU/ml) or 1/2× MIC of levofloxacin (7.44 ± 0.16 log CFU/ml) compared to controls. However, as it did for KS12, 1/2× MIC of minocycline again significantly reduced the titer of phage KS14 (6.11 ± 0.29 log CFU/ml; *P* < 0.05) compared to control conditions without antibiotics (7.31 ± 0.71 log CFU/ml).

The changes in phage plaque sizes were independent of the antibiotic resistance levels of the bacterial cells hosting phage production. Phage plaque size increases were tested in the presence of different concentrations of antibiotics on antibiotic-resistant bacterial cells. Compared to phage KS12 control conditions with no antibiotics, producing mean plaque diameters of 1.27 ± 0.09 mm on *B. cenocepacia* K56-2 cells harboring tetracycline-resistant plas-

TABLE 2 Plaque diameters of phages KS12 and KS14 in the presence of different concentrations of antibiotics

Phage and treatment or antibiotic (concn [μg/ml]) ^b	Mean plaque diam (mm) ± SD at antibiotic concn of ^a :					
	0	1/4× MIC	1/2× MIC	1× MIC	2× MIC	4× MIC
Phage KS12						
DLA	1.22 ± 0.12					
CIP (0.625–10)		1.33 ± 0.07**	1.45 ± 0.07****	1.51 ± 0.11****	1.82 ± 0.12****	2.23 ± 0.12****
MEM (2.5–40)		1.58 ± 0.08****	1.76 ± 0.11****	1.92 ± 0.26****	2.19 ± 0.26****	2.37 ± 0.15****
TET (2.5–40)		1.41 ± 0.31****	1.40 ± 0.32****	1.42 ± 0.32****	1.48 ± 0.08****	1.56 ± 0.11****
Phage KS14						
DLA	2.81 ± 0.23					
CIP (62.5–1,000)		3.20 ± 0.27***	3.66 ± 0.30****	3.67 ± 0.23****	3.77 ± 0.27****	3.88 ± 0.26****
MEM (2.5–40)		3.52 ± 0.24****	3.55 ± 0.27****	3.73 ± 0.40****	4.20 ± 0.39****	4.35 ± 0.50****
TET (25–100)		3.72 ± 0.28****	4.05 ± 0.30****	4.19 ± 0.22****	ND	ND

^a Plaque diameter values are the averages ± standard deviations for 20 different plaques from three separate trials. Statistical analysis was performed by using ANOVA with Dunnett’s posttest (****, *P* < 0.0001; ***, *P* < 0.001; **, *P* < 0.01). ND, not determined.

^b DLA, double-layer agar technique; CIP, ciprofloxacin; MEM, meropenem; TET, tetracycline.

TABLE 3 Titers of phages KS12 and KS14 in the presence of different concentrations of antibiotics

Phage and treatment or antibiotic (concn [$\mu\text{g/ml}$]) ^b	Mean phage titer (log CFU/ml) \pm SD at antibiotic concn of ^a :				
	0	1/4 \times MIC	1/2 \times MIC	1 \times MIC	2 \times MIC
Phage KS12					
DLA	5.99 \pm 0.34				
CIP (0.625–5)		6.02 \pm 0.34	6.29 \pm 0.41	6.32 \pm 0.33	6.37 \pm 0.27
MEM (2.5–20)		6.55 \pm 0.46	6.54 \pm 0.25	6.84 \pm 0.74	6.85 \pm 0.25
TET (2.5–20)		6.05 \pm 0.30	6.13 \pm 0.25	6.13 \pm 0.30	5.97 \pm 0.05
Phage KS14					
DLA	7.31 \pm 0.71				
CIP (62.5–500)		8.55 \pm 0.56**	8.75 \pm 0.43**	8.84 \pm 0.19**	8.81 \pm 0.30**
MEM (2.5–20)		9.01 \pm 0.29***	9.34 \pm 0.28***	8.97 \pm 0.25***	9.61 \pm 0.27***
TET (25–100)		8.97 \pm 0.74***	8.58 \pm 0.23**	8.71 \pm 0.41**	ND

^a Plaque diameter values are the averages \pm standard deviations for 20 different plaques from three separate trials. Statistical analysis was performed by using ANOVA with Dunnett's posttest (****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$). ND, not determined.

^b DLA, double-layer agar technique; CIP, ciprofloxacin; MEM, meropenem; TET, tetracycline.

mid pBBR1MCS-3, tetracycline-resistant K56-2 cells still showed increased KS12 plaque sizes in the presence of 1 \times MIC of meropenem (2.21 ± 0.19 mm; $P < 0.0001$) or 4 \times MIC of tetracycline (1.58 ± 0.09 mm; $P < 0.0001$). A *B. cenocepacia* K56-2 mutant expressing spontaneously acquired tetracycline resistance similarly showed increases in phage KS12 mean plaque sizes in the presence of antibiotics: 1.20 ± 0.06 mm for the no-antibiotic control, 1.62 ± 0.10 mm at 4 \times MIC of tetracycline ($P < 0.0001$), and 2.16 ± 0.24 mm at 1 \times MIC of meropenem ($P < 0.0001$).

Effects of subinhibitory concentrations of antibiotics on host morphology and growth. *B. cenocepacia* C6433 and K56-2 cells were visualized by using transmission electron microscopy in order to observe changes in cell morphology when cells were grown in the presence of subinhibitory concentrations of ciprofloxacin, meropenem, and tetracycline. As shown in Fig. 1, both C6433 and K56-2 cells appear filamentous in the presence of subinhibitory concentrations of ciprofloxacin, exist as chains of elongated cells in the presence of subinhibitory concentrations of

meropenem, and exist as clusters of cells in the presence of tetracycline. Subinhibitory concentrations of antibiotics were added to exponential-phase cells *in vitro* at 1/2 \times MIC, and growth was monitored over time. Cells exposed to subinhibitory concentrations of antibiotics were determined to exhibit no growth rate defects compared to controls containing no antibiotics (data not shown). There was an increase in cell concentrations of both C6433 and K56-2 by 1.5 logs over 6 h in the presence of 1/2 \times MIC of either ciprofloxacin, meropenem, or tetracycline. In contrast, K56-2 numbers *in vitro* decreased at 330 min with the addition of phage KS12, as shown in Fig. 2, and decreased dramatically with the addition of KS12 in the presence of 1/2 \times MIC of either ciprofloxacin, meropenem, or tetracycline.

Treatment of *B. cenocepacia*-infected *Galleria mellonella* larvae with a combination of phage and antibiotics. As shown in Fig. 3, *G. mellonella* larvae exhibited increased survival when treated with a combination of phage and antibiotics compared with the no-antibiotic or no-phage controls. At 48 h postinfection,

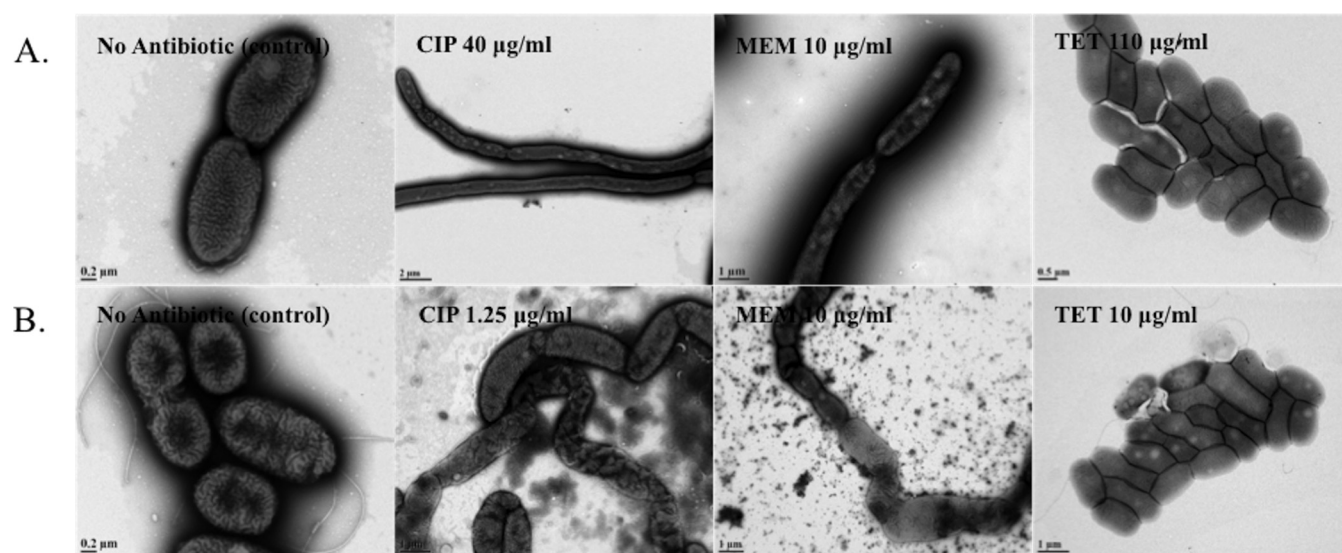


FIG 1 Transmission electron micrographs of *B. cenocepacia* strains C6433 (A) and K56-2 (B) in the presence of 1.25 $\mu\text{g/ml}$ ciprofloxacin (CIP), 5 $\mu\text{g/ml}$ meropenem (MEM), and 5.5 $\mu\text{g/ml}$ tetracycline (TET). Cells were stained with 2% phosphotungstic acid and imaged by using an FEI/Philips (Hillsboro, OR) Morgagni transmission electron microscope with a Gatan digital camera.

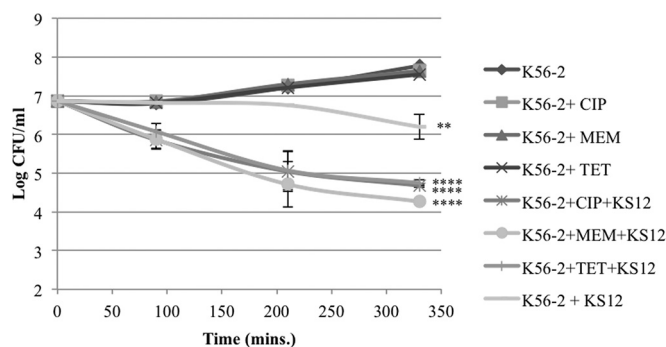


FIG 2 Killing effect of phage KS12 on *Burkholderia cenocepacia* K56-2 log-phase cells in the presence of ciprofloxacin (CIP), meropenem (MEM), and tetracycline (TET). Cells were treated with 1.25 μ g/ml ciprofloxacin, 5 μ g/ml meropenem, or 5.5 μ g/ml tetracycline. Phage KS12 was added at an MOI of 1. Values given are averages \pm standard deviations from three replicates. Statistical analysis was performed by using one-way ANOVA with Dunnett's post-test. **, $P < 0.01$; ****, $P < 0.0001$ (compared to the K56-2 control).

the mortality rate of larvae decreased from 80% when treated with meropenem alone, or 67% when treated with phage KS12 alone, to 22% upon treatment with phage KS12 and meropenem together. A similar decrease was observed with minocycline and phage KS12 *in vivo*: the larval mortality rate at 48 h was 76% with minocycline alone but only 31% when treatments with phage KS12 and minocycline were combined. These results are statistically significant ($P < 0.0001$). After 72 h, similar statistically significant results were observed ($P = 0.004$). For meropenem, there was a decrease in the mortality rate from 97% for KS12 alone to 57% for KS12 plus meropenem, and for minocycline, there was a decrease in the mortality rate from 100% with minocycline alone to 60% for KS12 plus minocycline. The rescue of *G. mellonella* with the addition of a subinhibitory concentration of meropenem and KS12 is similar to that with minocycline and KS12. In contrast, the addition of both meropenem and minocycline showed only minor improvement (61% mortality at 48 h) over either of the antibiotics alone (76% mortality for minocycline alone at 48 h versus 80% mortality for meropenem alone).

DISCUSSION

Members of the Bcc are known to have exquisite innate resistance to chemical antibiotics. This is attributed to several different mechanisms, including a number of biodegradative gene clusters on its large, multireplicon, ~ 8 -Mb genome. *B. cenocepacia* is inherently resistant to several different classes of antibiotics, including aminoglycosides, polymyxins, and most β -lactams (8), and can develop increased resistance to many drugs following repeated exposure. One form of resistance to aminoglycosides is typical of most bacteria, through aminoglycoside-inactivating enzymes, but the Bcc also has an amino-arabinose-modified lipopolysaccharide layer that prevents the entry of certain antibiotics and antimicrobial peptides (1, 46). Also, Bcc bacteria have multiple efflux pumps that remove antibiotics from the cell, thereby providing protection against various antibiotics (47, 48). Nevertheless, several antibiotics have been used in the treatment of Bcc infections. Many antibiotics belonging to different classes were tested for efficacy individually as well as in combination. Antibiotics with synergistic activity were identified, with the most effective combinations containing meropenem, which showed efficacy

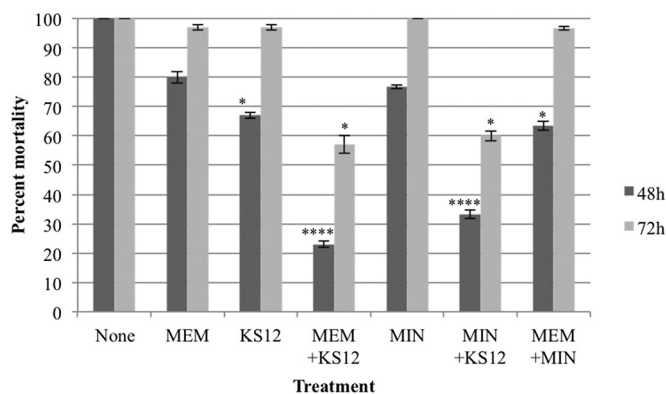


FIG 3 Mortality of *B. cenocepacia* K56-2-infected *G. mellonella* larvae treated with phage, antibiotic, or a combination of both. Larvae were infected with *B. cenocepacia* K56-2 ($9 \times$ the 50% lethal dose [9,000 CFU]) and treated with 6.0 μ g/ml meropenem (MEM) or 30 μ g/ml minocycline (MIN) alone or in combination with phage KS12 (MOI = 100). Values given are averages \pm standard deviations of data from three trials ($n = 10$ larvae per trial). Statistical analysis was performed by using one-way ANOVA with Dunnett's posttest. *, $P < 0.05$; ****, $P < 0.0001$ (compared to no-treatment controls).

in 70% of 119 isolates tested (19). However, 30% of Bcc strains tested retained resistance to the most active triple-combination synergistic antibiotic treatments, indicating that alternative therapies are desperately needed.

In this study, we have examined two strains of *B. cenocepacia*, strains C6433 and K56-2, since *B. cenocepacia* is one of the most prevalent Bcc species associated with CF infections (7). Subinhibitory concentrations of antibiotics have been shown to produce an increase in phage activity (26–35), and recently, this was termed “phage-antibiotic synergy,” or PAS (33). In this study, we have tested ciprofloxacin (a fluoroquinolone antibiotic), meropenem (a carbapenem, which is a modified β -lactam antibiotic), and tetracycline (a protein translation inhibitor). Although belonging to different classes of antibiotics and having different mechanisms of action, all three antibiotics exhibited PAS with phages KS12 and KS14, as detected by an enlargement in plaque size. Comeau et al. (33) suggested that PAS could be attributed to a change in bacterial cell morphology in the presence of subinhibitory concentrations of antibiotics, which permits rapid phage maturation and accelerated cell lysis. We performed transmission electron microscopy on Bcc cells in the presence of antibiotics at sub-MICs and found the cells to be elongated in the presence of ciprofloxacin and meropenem and in clusters in the presence of tetracycline. Cell filamentation was previously observed for subinhibitory concentrations of fluoroquinolones (49) and β -lactams (50). Our data confirm previously reported findings that phages may have increased access to phage receptors on elongated or filamentous cells, which leads to increased phage production and accelerated time to lysis. However, we also note that PAS is observed in the presence of subinhibitory concentrations of tetracycline, which causes cell clustering but not filamentation, indicating that filamentation is not a requirement for PAS. Instead, tetracycline PAS may suggest that cell clustering provides increased phage infection due to an ability of phages to travel laterally across adjoined cell surfaces, thereby enhancing contact with phage receptors on different cells.

Interestingly, there were differences between the PASs exhibited by each phage. Whereas both phages KS12 and KS14 displayed increased mean plaque diameters in the presence of differ-

ent antibiotics (Table 2), only KS14 appeared to exhibit greatly increased phage titers when exposed to different antibiotics (Table 3). At this time, we have no explanation for this effect. A similar riddle exists for differences between antibiotics. Both phages exposed to minocycline produced increased mean plaque diameters, but only minocycline also significantly reduced both phage titers produced in each plaque. Curiously, in the *G. mellonella* infection model, minocycline was almost as effective at reducing larval mortality in combination with phage KS12 as meropenem at both 48 and 72 h (although meropenem was used at a sub-MIC of 6 µg/ml, whereas minocycline was used at 30 µg/ml). This suggests that despite the lower numbers of phages being produced through PAS by minocycline, there are still sufficient phage numbers to produce a therapeutic PAS effect *in vivo*. Somewhat surprisingly, a related drug like tetracycline, with a chemical structure and a mechanism of action similar to those of minocycline, produces “normal” PAS, with an increased plaque diameter and without an apparent reduction in phage titers.

Phage therapy against Bcc infections has produced promising results both in *G. mellonella* larvae (22) and when delivered by aerosol into lungs in a mouse infection model (26). In this study, we have combined phage therapy with doses of antibiotics to observe PAS in the treatment of a Bcc infection in *G. mellonella*. Our results show larval rescue from Bcc infection even at very low MOIs of KS12, with larva mortality falling dramatically from 65% at 48 h when treated with KS12 alone to 20% at 48 h when treated with a combination of KS12 and meropenem. These results are similar to those reported previously for the treatment of *P. aeruginosa*; cells treated with a combination of phage and antibiotic showed better survival than those treated with phage alone (34). Moreover, both minocycline and meropenem with KS12 gave similar results *in vivo*, suggesting that PAS is operating similarly, even though the antibiotics’ mechanisms of action on the bacterial cells are different. Studies with the objective to better understand the mechanisms of action behind PAS are under way in our laboratory.

Interestingly, the antibiotic resistance status of the target cell does not change PAS. When we made cells artificially tetracycline resistant, either through mutation or transformation with a tetracycline-resistant plasmid, PAS was not altered. This finding suggests that whatever the effects that subinhibitory levels of antibiotics are having on the cell to make them more sensitive to phages, they are not related to the cell’s innate or acquired antibiotic resistance levels. It is encouraging to note, therefore, that PAS could still be used for treatment of cells that have previously become resistant to antibiotic treatment. Given that members of the Bcc cannot be cleared from the lungs of patients with cystic fibrosis by using antibiotics, almost all antibiotic treatments of these patients are subinhibitory (depending upon the Bcc strain), yet PAS appears to offer a realistic way to reduce bacterial numbers below those achieved by either antibiotic or phage treatment alone.

With the emergence of antibiotic resistance, phage therapy in combination with antibiotics may be useful as an alternative treatment. Members of the Bcc have a high level of innate antibiotic resistance, which is in part due to their ability to form biofilms (12). As some phages have been shown to readily penetrate bacterial biofilms (51–53), it is anticipated that PAS will work as well against bacteria growing in biofilms as planktonic bacteria. In support of this hypothesis, PAS has been shown to work well against *E. coli* biofilms, as evidenced by a decrease in the minimum bio-

film eradication concentration (MBEC) of the antibiotic cefotaxime when used in combination with phage T4 (35). Thus, combining phage therapy with traditional antibiotics could help better manage antibiotic-resistant bacterial infections, regardless of whether or not the cells are growing in bacterial biofilms.

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